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(71) Applicant: Standard Oil Company, 200 East Randolph
Drive, Chicago Illinois 60680 (US)

(72) Inventor: Heller, Michael James, 30 W 057-104 Granada,
Naperville Illinois 60540 (US)
Inventor: Morrison, Larry Edward, 4913 Spencer, Lisle
Illinois 60532 (US)
Inventor: Prevatt, William Dudley, 1205 Grant Street,
Downers Grove Illinois 60515 (US)
Inventor: Akin, Cavit, 1462 Inverrary Drive, Naperville
Illinois 60540 (US)

(74) Representative: Ritter, Stephen David et al, Mathys &
Squire 10 Fleet Street, London EC4Y 1AY (GB)

(54) Light-emitting polynucleotide hybridization diagnostic method.

(57) A hybridization diagnostic method is disclosed which
uses light-labeled, single-stranded polynucleotide reagents for
hybridizing with immobilized sample single-stranded polynu-
cleotides. Light emission is related to the amount of target po-
lynucleotide in the sample.

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LIGHT-EMITTING POLYNUCLEOTIDE
HYBRIDIZATION DIAGNOSTIC METHOD

BACKGROUND OF THE INVENTION

5   Presently, nucleic acid hybridization assays
are primarily used as a research tool for the detec-
tion and identification of a unique deoxyribonucleic
acid (DNA) sequence or a specific gene in a complete
DNA, a mixture of DNA's, or a mixture of DNA frag-
10   ments. A number of variations of the technique
exist as used in recombinant DNA research (Methods
in Enzymology, Vol. 68, R. Wu (Ed.) pp 379-469,
1979; and Dunn, A. R., and Sambrook, J., Methods in
Enzymology, Vol. 65, Part 1, 468-478, 1980). One
15   of the most widely used procedures is called the
Southern blot filter hybridization method (Southern,
E., J. Mol. Biol. 98, 503, 1975). This procedure
is usually used to identify a particular DNA frag-
ment separated from a mixture of DNA fragments by
20   electrophoresis. The procedure is generally carried
out by subjecting a sample of DNA isolated from
some organism to restriction endonuclease digestion
and then electrophoresing the sample on a gel
(agarose, acrylamide, etc.). The gel containing
25   the separated DNA fragments is placed in contact
(blotted) with a nitrocellulose filter sheet (or
diazotized paper, etc.). The DNA fragments are
transferred and become bound to the nitrocellulose
sheet. The gel-transfer nitrocellulose sheet con-
30   taining the DNA fragments is then heated (95°C) to
denature the DNA. At this point the sheet is treated
with a solution containing a denatured radio labeled
(^{32}p) "specific DNA probe" and hybridization is
allowed to take place. Hybridization can take from
35   3-48 hours, depending on given conditions, and un-
hybridized "specific DNA probe" is washed away.
The nitrocellulose sheet is then placed on a sheet

of X-ray film and allowed to expose, usually for several days at -70°C. The X-ray film is then developed. The exposed areas on the film identify which DNA fragments have hybridized and thus have a sequence similar to that of the "specific DNA probe."

This procedure, as well as most other variations, requires the use of radioisotopes and is obviously very complex and time consuming. Because of these and other problems, DNA hybridization assays have remained only as a tool for basic research and have not been generally used in applied or commercial areas, such as for clinical diagnostics.

It would be very desirable to have a hybridization method which would avoid some of these problems, particularly the use of radioisotopes. Hybridization assays could be a valuable diagnostic method for identification of pathogenic organisms and for the determination of antibiotic resistant traits (genes) in microorganisms. They could also be used for genetically based human cell typing systems and for diagnosis of genetic diseases.

SUMMARY OF THE INVENTION

In general, the invention relates to a hybridization assay which is based on the inherent high fidelity of the base recognition process in complementary double-stranded (ds) nucleic acids (DNA, RNA, DNA-RNA, and synthetic polynucleotides). It involves a hybridization system that does not use radioisotopes, but instead uses chemiluminescent, fluorescent, or phosphorescent moieties which under proper conditions can provide sensitivity equal to that of radioisotopes but without the inherent problems. This invention has advantages over present methods which are too complex and time consuming to be used practically in clinical diagnostics.

In one aspect, the invention resides in a diagnostic method for determining the presence of and

the identification of viruses, bacteria, and other
microorganisms, as well as the existence of certain
genetic traits by assaying for a target nucleotide
sequence which is uniquely characteristic of the
5   target microorganism or genetic trait being assayed.

In particular, the method comprises the steps
of (a) immobilizing the sample single-stranded poly-
nucleotides on a suitable support; (b) contacting
the immobilized sample, under hybridization condi-
10   tions, with light-labeled reagent single-stranded
polynucleotide segments which are complementary to
a representative portion of the target single-stranded
polynucleotide; (c) separating unhybridized reagent
single-stranded polynucleotide segments from the
15   immobilized sample; (d) exposing the immobilized
sample to means for exciting the light label; and
(e) detecting the light response from the immobilized
sample.

The methods of this invention are directed towards
20   the single-stranded polynucleotides because of their
ability to combine with complementary strands in a
highly specific manner. The single-stranded poly-
nucleotides useful for purposes of this invention
are readily obtained from double-stranded polynucleo-
25   tides by denaturing, i.e. splitting the double-
stranded polynucleotide into two complementary
strands. This is typically accomplished by heating
to about 70-100°C for about 5-10 minutes. The
"target" single-stranded polynucleotide mentioned
30   above is either of the two single-stranded polynucleo-
tides obtained by denaturing that double-stranded
polynucleotide (the target double-stranded polynucleo-
tide) at which the diagnosis is ultimately directed,
such as a particular gene. Similarly, the "single-
35   stranded polynucleotide sample" can be obtained by
isolating the double-stranded polynucleotides from
the physiological sample to be diagnosed (blood,

urine, other body fluids, etc.) and denaturing the double-stranded polynucleotides to form the corresponding single strands.

5   Immobilization of the sample single-stranded polynucleotides can be accomplished by any suitable method which does not inactivate a significant number of the bases in the polynucleotide sequence, since a representative intact sequence must be available for base pairing with the reagent strands. The

10   single-stranded polynucleotide segment can be attached or immobilized to a variety of supports, some of which include activated glass beads, polyacrylamide, agarose or sephadex beads, and cellulose. Numerous methods exist for coupling either terminal end of a

15   polynucleotide to a given support (Weissback, A., and Poonian, M., Methods in Enzymology, Vol. XXXIV, Part B, 463-475, 1974). Also, derivatized forms of polynucleotides, for example, one carrying a terminal aminohexyl nucleotide, can be easily attached on a

20   variety of supports (Mosbach, K., et al., Methods in Enzymology, Vol. XLIV, 859-886, 1976). Oligoribonucleotides may be immobilized on boronate derivates of various supports (Schott, H., et al., Biochemistry, 12, 932, 1973).

25   Hybridization conditions necessary to accomplish base pairing of the target single-stranded polynucleotide in the sample (if present) and the reagent single-stranded polynucleotide segments are determined by a variety of factors, such as the nature of the

30   light label attached to the reagent polynucleotide sequences, the size of the reagent polynucleotide sequences, the [G] + [C] (guanine plus cytosine) content of the reagent and sample polynucleotide sequences, and how the sample polynucleotide sequence

35   is prepared. The light label can affect the temperature and salt concentration for the hybridization reaction. Chemiluminescent catalysts can be sensi-

tive to temperatures and salt concentrations that
absorber/emitter moieties can tolerate. Size of
the reagent polynucleotide sequences affects the
temperature and time for the hybridization reaction.

5 Assuming similar salt and reagent concentrations,
hybridization involving reagent polynucleotide se-
quences in the range of 10,000 to 100,000 nucleotides
might require from 40 to 80 minutes to occur at
67°C, while hybridization involving 20 to 100 nucleo-

10 tides would require from 50 to 30 minutes at 25°C.
Similarly, the [G] + [C] content of the reagent and
sample polynucleotide sequences affects the tempera-
ture and time for the hybridization reaction. Poly-
nucleotide sequences with a high [G] + [C] content

15 will hybridize at lower temperatures in a shorter
period of time than polynucleotide sequences with a
low [G] + [C] content. Finally, conditions used to
prepare the sample polynucleotide sequence and main-
tain it in the single-stranded form can affect the

20 temperature, time, and salt concentration of the
hybridization reaction. The conditions for preparing
the sample polynucleotide sequence are affected by
polynucleotide length required and the [G] + [C]
content. In general, the longer the sequence or

25 the higher the [G] + [C] content, the higher the
temperature and/or salt concentration required.
      Reagent single-stranded polynucleotide segments
must be complementary to a representative portion
of the target single-stranded polynucleotide so as

30 not to give a false indication of a positive test
in the absence of the target single-stranded poly-
nucleotide in the sample. The term "representative"
is intended to be an imprecise term since the desired
accuracy of the method will vary with the purpose.

35 Ideally, the reagent single-stranded polynucleotide
will only pair with target single-stranded polynucleo-
tide and will do so whenever the latter is present

in the sample. If the target single-stranded poly-
nucleotide base sequence is very unique, very high
accuracy can be obtained. Preferably, the reagent
single-stranded polynucleotide segments are prepared
5   from the target double-stranded polynucleotide,
which is denatured and separated into the two com-
plementary single strands. Either single strand
can be chosen as the reagent material to be light-
labeled, but only one of them can actually be used
10   in order to prevent the reagent from hybridizing
with itself. The sample single-stranded polynucleo-
tides need not be separated in this manner, however,
in which case only half of the single-stranded poly-
nucleotides present in the sample, which were derived
15   from the target double-stranded polynucleotide,
would be suitable hybridization candidates. If
sequences are known, then reagent single-stranded
polynucleotides can be made by organic synthetic
techniques.

20   Light labels can be chemiluminescent, biolumines-
cent, phosphorescent, or fluorescent and can be
attached to any point on the reagent single-stranded
polynucleotide segments; however, terminal positions
are more desirable. Suitable light labels of the
25   chemiluminescent type include, without limitation,
peroxidase and iron porphyrin derivatives. Suitable
light labels of the   bioluminescent type include,
without limitation, bacterial luciferase (either
one or both luciferase and associated dehydrogenase),
30   firefly luciferase, flavin mononucleotide (FMN),
adenosine triphosphate (ATP), reduced nicotinamide
adenine dinucleotide (NADH), reduced nicotinamide
adenine dinucleotide phosphate (NADPH), and various
long chain aldehydes (decyl aldehyde, etc.). Suit-
35   able fluorescent light labels include, without limita-
tion, a variety of fluorescent nucleotides (etheno-
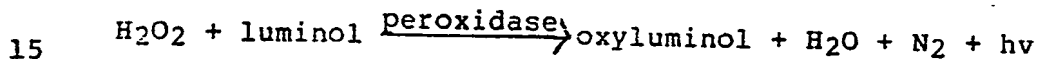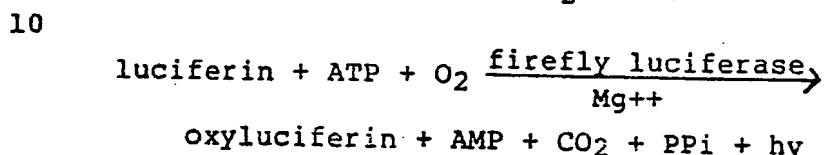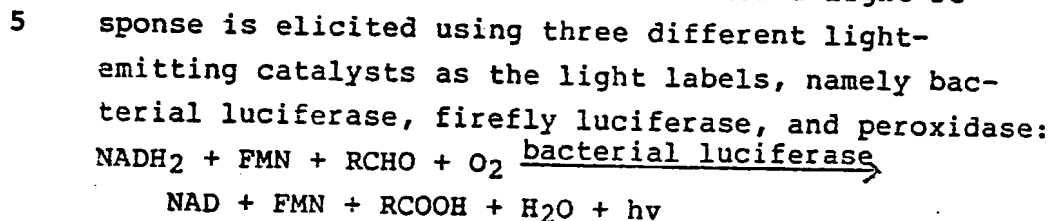adenosine nucleotides, etheno-cytidine nucleotides,

etc.) or functionalized nucleotides (amino-hexane
adenosine nucleotides, mercurinucleotides, etc.)
which could first be fluorescently labeled and then
covalently attached to the reagent single-stranded

5   polynucleotide segment. A variety of methods exist
for preparing fluorescent derivatives of oligoribo-
nucleotides (Annual Review Biophys. Bioeng., A. Faure
and G. Thomas, pp. 175-195, 1981). Suitable phos-
phorescent light labels include, without limitation,

10   2-diketones such as 2,3-butadione, 1-[carboxyphenyl]-
1,2-pentanedione, and 1-phenyl-1,2-propanedione.
Means for attaching the light labels to the reagent
single-stranded polynucleotide segments are documented
in the art.

15   In order to determine if hybridization has occur-
red and hence the presence of the target polynucleo-
tide in the sample, it is first necessary to remove
the unhybridized light-labeled reagent. This is
most simply accomplished by washing or flushing

20   with appropriate buffer solution. Complete removal
of unhybridized reagent is a goal to be attained,
but is not likely since there will be some residual
reagent which sticks to the immobilization support
or possible partially hybridizes with the sample.

25   These occurrences will cause "background" when measur-
ing the light response and must be accounted for
when analyzing the results of the test.

           After the separating step, the immobilized sample
must be exposed to a means for exciting the light

30   labels, if present, which may have become immobilized
through hybridization between the reagent and immobil-
ized sample single-stranded polynucleotides. In
the case of fluorescent or phosphorescent labels,
the sample need only be irradiated with the appropri-

35   ate light wavelength. This technique is well known
in the art. In the case of the chemiluminescent or
bioluminescent labels, the sample must be exposed

to a light reagent system which depends specifically on the light label which has been used. Such light reagents are also well known in the art. For example, the following reactions illustrate how a light response is elicited using three different light-emitting catalysts as the light labels, namely bacterial luciferase, firefly luciferase, and peroxidase:

$$NADH_2 + FMN + RCHO + O_2 \xrightarrow{\text{bacterial luciferase}}$$
$$NAD + FMN + RCOOH + H_2O + hv$$

$$luciferin + ATP + O_2 \xrightarrow[Mg++]{\text{firefly luciferase}}$$
$$oxyluciferin + AMP + CO_2 + PPi + hv$$

$$H_2O_2 + luminol \xrightarrow{\text{peroxidase}} oxyluminol + H_2O + N_2 + hv$$

In the presence of an excess of reactants, the light response will vary with the number of light-labeled reagent single-stranded polynucleotide segments which have become hybridized with the immobilized sample. The choice of the label will be determined by ease of labeling and the economics of the light reagent system.

The detection of the light response can be accomplished with numerous detection devices which are commercially available. For example, using photomultiplier detection devices, $NADH_2$ levels of $10^{-3}-10^{-9}$ mg./ml., ATP levels of $10^{-3}-10^{-11}$ mg./ml., and H_2O_2 levels of $10^{-3}-10^{-7}$ mg./ml. can be measured.

As mentioned above, it is necessary to immobilize the sample single-stranded polynucleotide on a support. In addition to the aforementioned methods of immobilization, the sample single-stranded polynucleotide may be immobilized by contacting the sample under hybridization conditions with an immobilized single-stranded polynucleotide reagent segment, said first single-stranded polynucleotide reagent segment being complementary to a portion of the target single-stranded polynucleotide. When target

single-stranded polynucleotide is present in the
sample it hybridized with the immobilized first
single-stranded polynucleotide reagent segment.
The target single-stranded polynucleotide then serves
as a template for hybridization with the light-
labeled second single-stranded polynucleotide reagent
segment which is complementary to a substantially
mutually exclusive portion of the target single-
stranded polynucleotide relative to said first single-
stranded polynucleotide reagent segment. This system
has the advantage of improved specificity because
it involves two hybridization processes.

The first and second single-stranded polynucleo-
tide reagent segments must consist essentially of
bases which are complementary to the base sequence
of the target single-stranded polynucleotide. It
is necessary that the first and second segments be
complementary to substantially mutually exclusive
portions of the target single-stranded polynucleotide.
In other words, upon hybridization with the target
polynucleotide the first and second reagent segments
should not compete for the same base sequence to
the extent that hybridization is prevented. In
some cases, the first and second segments will line
up head to tail (3' end to 5' end) with no overlap
and with few or no basepairing spaces left between
them. However, in other cases it will be highly
desirable (because it would improve specificity)
that the first and second single-stranded polynucleo-
tide reagent segments be complementary to two widely
separated but unique sequences on the target single-
stranded polynucleotide.

First and second single-stranded polynucleotide
reagent segments can be prepared from appropriate
restriction endonuclease treatment of target nucleic
acids from the organism of interest or, in cases
where the base sequence of a unique portion is known,

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they can also be synthesized by organic synthetic techniques (Stawinski, J., et al., Nuc. Acids Res. 4, 353, 1977; Gough, G. R. et al., Nuc. Acids Res. 6, 1557, 1979; Gough, G. R. et al., Nuc. Acids Res. 7, 1955, 1979; Narang, S. A., Methods in Enzymology, Vol. 65, Part I, 610-620, 1980).

The size of the reagent segments can be from 10 nucleotides to 100,00 nucleotides in length. Hybridized systems having fewer than 10 nucleotides are not stable and will begin to denature about 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much slower and incomplete process, (See Molecular Genetics, G. S. Stent and R. Calender, pp 213-219, 1971). Ideally the reagent segments should be from 20 to 10,000 nucleotides. Smaller nucleotide sequences (20-100) would lend themselves to production by automated organic synthetic techniques. Sequences from 100-100,000 nucleotides could be obtained from appropriate restriction endonuclease treatments. Ideally small sequences should be fluorescent labeled, while larger sequences could be chemiluminescent labeled. The labeling of the smaller segments with the relatively bulky chemiluminescent moieties may in some cases interfere with the hybridization process.

It will be appreciated by those skilled in the art that many variations from the foregoing can be made without departing from the scope of this invention.

We claim:

1.　A method for assaying target single-stranded polynucleotides in a single-stranded polynucleotide sample comprising:

5　　　　　a)　contacting an immobilized sample single-stranded polynucleotide on a support, under hybridization conditions, with light-labeled reagent single-stranded polynucleotide segments which are complementary to a representative portion of the target single-

10　stranded polynucleotide;

　　　　　b)　separating unhybridized reagent single-stranded polynucleotide segments from the immobilized sample,

　　　　　c)　exposing the immobilized sample to

15　means for exciting the light labeled; and

　　　　　d)　detecting the light response from the sample.

2.　The method of Claim 1 wherein (a) the sample single-stranded polynucleotides are immobilized by

20　contacting the sample, under hybridization conditions, with immobilized first single-stranded polynucleotide reagent segments being complementary to a portion of the target single-stranded polynucleotides, and (b) the light-labeled reagent single-stranded poly-

25　nucleotide segments are light-labeled second single-stranded polynucleotide reagent segments which are complementary to a substantially mutually exclusive portion of the target single-stranded polynucleotide relative to said first single-stranded polynucleotide

30　reagent segment.

3.　The method of Claim 1 or Claim 2 wherein the light label is a chemiluminescent label.

4.　The method of Claim 3 wherein the chemiluminescent label is selected from the group consisting

35　of peroxidase and iron porphyrin derivatives.

5.　The method of Claim 1 or Claim 2 wherein the light label is a fluorescent label.

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6.   The method of Claim 5 wherein the fluorescent label is selected from the group consisting of etheno-adenosine nucleotides, etheno-cytidene nucleotides, amino-hexane adenosine nucleotides, and mer-
5   curinucleotides.

7.   The method of Claim 1 or Claim 2 wherein the light label is a bioluminescent label.

8.   The method of Claim 7 wherein the bioluminescent label is selected from the group consisting of
10   bacterial luciferase, firefly luciferase, flavin mononucleotide, adenosine triphosphate, reduced nicotinamide adenine dinucleotide, reduced nicotinamide adenine dinucleotide phosphate, and decyl aldehyde.

15   9.   The method of Claim 1 or Claim 2 wherein the light label is a phosphorescent label.

10.   The method of Claim 9 wherein the phosphorescent label is selected from the group consisting of 2,3-butadione, 1-[carboxyphenyl]-1,2-pentanedione,
20   and 1-phenyl-1,2 propanedione.